Efflux pump expression in MDR *Acinetobacter baumannii* strains from a tertiary care hospital in Lima, Peru

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Research note

Keywords: Acinetobacter baumannii, Efflux Pump Inhibitors, Antimicrobial Resistance, Multidrug Resistance

Posted Date: May 22nd, 2020

DOI: https://doi.org/10.21203/rs.3.rs-25924/v1

Abstract

Objetive: To analyze the contribution of the active efflux system to quinolones and aminoglycosides resistance in selected outbreak *A. baumannii* clinical isolates using the efflux pump inhibitor PAβN.

Results: A total of nineteen *Acinetobacter baumannii* strains were included in the study. All were positive for the *bla*OXA-51 gene by PCR and had clinical information associated. The samples were non-duplicate and collected from different sources. Non-susceptibility rates were as following: tobramycin 31.6% (6), ciprofloxacin 31.6% (6), levofloxacin 21.1% (4), nalidixic acid 26.3% (5) and amikacin 15.8% (3). A total of eight strains (42,1%) demonstrated an increase in the susceptibility rates and sixteen (84,2%) expressed efflux pumps.

Introduction

Acinetobacter spp. comprehend gram-negative coccobacilli, non-fermentators, non-motile, oxidase negative, catalase positive and correspond to Moraxellaceae family(1). *A. baumannii, Acinetobacter calcoaceticus-Acinetobacter baumannii* complex (ACB), *A. nosocomialis* and *A. pitti* comprehend an 90 to 95% of clinically significative infections (2).

A. baumannii is an emerging nosocomial pathogen that has developed mechanisms to resist disinfection, desiccation and oxidative stress (3). Its ability to survive under a wide range of environmental conditions and to persist for extended periods of time on surfaces makes it a frequent cause of outbreaks and an endemic healthcare–associated pathogen (4), whose clinical significance has been increasing in the last three decades (5). Institutional outbreaks caused by multidrug-resistant (MDR) strains are a growing public-health concern (1).

A. baumannii causes ventilator-associated pneumonia or central-line bloodstream infections and less frequently skin and soft tissues infections, surgical site infection and catheter-associated urinary tract infections (5, 8–10). Risk factors for colonization or infection with multidrug-resistant species include prolonged hospitalization, admission at the intensive care unit (ICU), mechanical ventilation, long-term exposure to broad-spectrum antibiotics, recent surgery, invasive procedures and underlying severe illness (1, 4, 6–11).

Several intrinsic and acquired resistance mechanisms are expressed frequently in nosocomial strains, including: increased production of antibiotic efflux pumps(12), point mutations in target proteins to inactivate antibiotic effect, enzymatic modification, antimicrobial degradation and reduction of membrane permeability (13). To cause clinical resistance in *Acinetobacter*, efflux pumps usually act in association with overexpression of Amp C β-lactamases or carbapenemases. In addition to removing β-lactam antibiotics, efflux pumps can actively expel macrolides, quinolones, tetracyclines, chloramphenicol and disinfectants (11).

Efflux pumps usually have 3 components and *A. baumannii* may contain more than six different transporter superfamilies capable of actively pumping out a broad range of antimicrobial and toxic compounds from the cell (14). Five distinct families of transport proteins have been shown to include multidrug efflux systems (14). Recently, the proteobacterial antimicrobial compound efflux (PACE) family has been described as a sixth family of bacterial multidrug efflux systems (14). RND-type transporters in particular are known to play a dominant role in the MDR of many *Acinetobacter* species(15). While the overexpression of Ade transporters is often beneficial to bacteria, this is not always the case; some Ade transporters, such as AdeABC, AdeFGH, and AdeIJK, can be toxic to cells when overexpressed (16, 17). To assess the role of drug efflux mechanism in bacteria, efflux pump inhibitors (EPIs) are widely used (18). The effects of several EPIs, including carbonyl cyanide m-chlorophenylhydrazone (CCCP), phenyl-arginine-β- naphthylamide (PAβN) (19) and 1-(1-napthylmethyl)-piperazine (NMP) (20–22) has been assessed in a small number of in vitro studies, including CCCP, PABN(19) and NMP, along with other drugs that may impact efflux mechanisms (omeprazole, verapamil, reserpine, phenothiazines) (20–22). One of the best-studied EPIs is the peptidomimetic compound, phenylalanine-arginine b-naphthylamide (PABN, also called MC207, 110), which was originally described in 1999 and characterized in 2001 as a broad-spectrum efflux pump inhibitor, capable of significantly reducing fluoroquinolone resistance (23) and permeabilizing membranes in *P. aeruginosa* (24).

Hence, the aim of this study is to analyze the contribution of the active efflux system to quinolones and aminoglycosides resistance in selected outbreak *A. baumannii* clinical isolates using the efflux pump inhibitor PAβN.

Material And Methods

Samples

This study included nineteen non-duplicate clinical samples identified as *Acinetobacter* spp. by BD Phoenix™ Automated Microbiology System (BD Biosciences, USA) and collected from inpatients at Instituto Nacional de Enfermedades Neoplásicas (INEN) over a 24-months period (January 2014-December 2016) in Lima, Peru. Patients characteristics were collected from clinical records and were coded to avoid identification.

Isolates were obtained from blood, bronchial aspirate, soft tissues, cerebrospinal fluid and urine. Samples were stored and frozen at -80 °C, collected as routine internal surveillance programs.

Strains were recovered as part of an outbreak study (25) and selected MDR strains were included and further assessed by microbiological and molecular techniques at the Molecular Biology Laboratory, *Universidad Peruana de Ciencias Aplicadas* (UPC) and *Instituto de Investigación Nutricional* (IIN).

Bacterial culture conditions and identification

Clinical samples were cultured in tryptic soy agar (TSA) at 37 °C for approximately 24 hours under aerobic conditions (26). The *A. baumannii* strain identification was confirmed by PCR of $bla_{\text{OXA-51-like}}$ gene (27). Amplified products were gel recovered, purified (SpinPrep™ Gel DNA Kit, San Diego, USA) and sent to be sequenced (Macrogen, Seoul, Korea).

All the bacteria isolated and included in the study are disposable for scientific non-commercial purposes.

Antimicrobial susceptibility testing

Antimicrobial susceptibility to quinolones (levofloxacin, ciprofloxacin) and aminoglycosides (tobramycin, amikacin) was assessed by broth microdilution technique, according to 2018 Clinical and Laboratory Standards Institute (CLSI) guidelines (28), and as described by Gholami et al (23), except nalidixic acid whose resistance values were assessed according to Clinical and Laboratory Standards Institute (CLSI) guidelines(29). MIC concentrations tested ranged from 0.25 µg/mL up to 256 µg/mL in a 96-well microtiter plate with 100 µL of the antibiotic dilution and Müller-Hinton broth. The correct density of the samples was standardized at 625 nm spectrophotometry, and then were incubated at 37 °C for 24 hours. The MIC was the lowest concentration without detection of bacterial growth(23, 28). *Escherichia coli* ATCC 25922 was used as a quality control strain.

Efflux Pump Inhibitor

The activity of the efflux pumps system was assessed by the addition of phenylalanine inhibitor of arginine-β-naphthylamide (PAβN) in broth microdilution, as previously assessed in the study of Gholami et al (23). Efflux pump expression was based on a fourfold or greater reduction in the MIC as the criterion for significance as described in the literature (21, 30).

Ethics Statement

The study protocol was approved by the Research Ethics Board of the *Instituto de Investigación Nutricional* (IIN). The samples of clinical laboratory isolates were obtained in the context of infection surveillance regulated by the Committee for the Control and Prevention of Intrahospital Infections of the *Instituto Nacional de Enfermedades Neoplásicas* in accordance with Technical Standard No. 753–2004 / Ministry of Health of Peru. and with the International Ethical Guidelines for Health-related Research Involving Humans. In-hospital infections (IIH) also called - Health care-associated infections (HCAIs) are a Public Health problem and under these provisions, the collection of samples was exempt from informed consent. Patient information was coded when it was collected to ensure anonymity and confidentiality and its characteristics were evaluated from their clinical records.

Results

A total of nineteen *Acinetobacter* spp. strains were included in the study, they were positive for *bla*OXA-51 gene by PCR, identifying them as *A. baumannii*.

MIC Susceptibility profiles of the strains included in this study are described in Table 1. Non-susceptibility rates were as following: tobramycin 31.6% (n = 6), ciprofloxacin 31.6% (n = 6), levofloxacin 21.1% (n = 4), nalidixic acid 26.3% (n = 5) and amikacin 15.8% (n = 3).

Table 1

Susceptibility profiles of the *Acinetobacter baumannii* strains included in this study[a].

Strain	MIC (µg/ml) (susceptibility rating) for indicated drug									
	Antibiotic					Antibiotic + PaβN				
	TOB	CIP	LEV	NA	AK	TOB	CIP	LEV	NA	AK
Ac2	0.5 (S)	0.5 (S)	8 (R)	128 (R)	< 0.25 (S)	1 (S)	0.25 (S)	**2 (S)[i]**	**32 (R)**	2 (S)
Ac10	1 (S)	0.5 (S)	32 (R)	32 (R)	< 0.25 (S)	2 (S)	1 (S)	32 (R)	32 (R)	1 (S)
Ac20	8 (I)	2 (I)	1 (S)	1 (S)	< 0.25 (S)	4 (S)	**0.5 (S)[i]**	4 (I)	4 (S)	< 0.25 (S)
Ac21	8 (I)	< 0.25 (S)	< 0.25 (S)	1 (S)	8 (S)	4 (S)	32 (R)	0.5 (S)	2 (S)	4 (S)
Ac22	0.5 (S)	0.25 (S)	2 (S)	16 (S)	4 (S)	0.25 (S)	0.5 (S)	4 (I)	**1 (S)**	2 (S)
Ac23	64 (R)	8 (R)	0.25 (S)	0.5 (S)	16 (S)	64 (R)	**2 (I)[i]**	0.25 (S)	2 (S)	**4 (S)**
Ac24	0.5 (S)	2 (I)	0.5 (S)	1 (S)	32 (I)	1 (S)	**0.25 (S)[i]**	0.25 (S)	0.5 (S)	16 (S)
Ac25	32 (R)	0.5 (S)	1 (S)	0.25 (S)	32 (I)	16 (R)	0.25 (S)	**0.25 (S)**	0.5 (S)	**4 (S)[i]**
Ac26	0.25 (S)	8 (R)	0.5 (S)	64 (R)	< 0.25 (S)	2 (S)	16 (R)	1 (S)	32 (R)	< 0.25 (S)
Ac27	0.5 (S)	1 (S)	0.25 (S)	64 (R)	16 (S)	4 (S)	2 (I)	0.25 (S)	**8 (S)[i]**	**4 (S)**
Ac28	0.25 (S)	4 (R)	2 (S)	16 (S)	< 0.25 (S)	4 (S)	**< 0.25 (S)[i]**	**0.5 (S)**	16 (S)	1 (S)
Ac29	0.5 (S)	2 (I)	1 (S)	64 (R)	32 (I)	4 (S)	2 (I)	1 (S)	32 (R)	**4 (S)[i]**
Ac30	2 (S)	0.5 (S)	1 (S)	2 (S)	0.5 (S)	**0.5 (S)**	0.25 (S)	**< 0.25 (S)**	**0.5 (S)**	0.25 (S)
Ac39	< 0.25 (S)	< 0.25 (S)	2 (S)	2 (S)	1 (S)	< 0.25 (S)	< 0.25 (S)	**0.5 (S)**	**0.25 (S)**	0.5 (S)
Ac41	16 (R)	< 0.25 (S)	1 (S)	4 (S)	2 (S)	16 (R)	< 0.25 (S)	2 (S)	2 (S)	**< 0.25 (S)**
Ac46	16 (R)	< 0.25 (S)	0.25 (S)	4 (S)	< 0.25 (S)	8 (I)	0.25 (S)	< 0.25 (S)	**1 (S)**	0.25 (S)
Ac50	1 (S)	< 0.25 (S)	< 0.25 (S)	0.25 (S)	16 (S)	0.5 (S)	4 (R)	< 0.25 (S)	0.25 (S)	**< 0.25 (S)**
Ac54	4 (S)	1 (S)	4 (I)	2 (S)	< 0.25 (S)	**1 (S)**	1 (S)	2 (S)	**0.25 (S)**	4 (S)
Ac55	4 (S)	0.5 (S)	4 (I)	2 (S)	1 (S)	4 (S)	0.5 (S)	4 (I)	2 (S)	**< 0.25 (S)**

[a] TOB, tobramycin; CIP, ciprofloxacin; LEV, levofloxacin; NA, nalidixic acid; AK, amikacin; R, resistant; S, susceptible; I, intermediate.

i: change of susceptibility interpretation according to CLSI 2018 MIC Values

Bold: four fold change in MIC

Efflux pump expression was determined by the measurement of MIC according to previously described parameters. A total of sixteen strains (84.2%) expressed measurements that confirmed efflux pumps presence and eight of them (42.1%) demonstrated an increase in the susceptibility profile, being the more frequent antimicrobial ciprofloxacin (n = 4; 21.1%). Considering only MIC reduction, more strains demonstrated changefor amikacin (n = 8; 42.1%) and nalidixic acid (n = 7; 36.8%), followed by levofloxacin (n = 5; 26.3%), ciprofloxacin (n = 4; 21.1%) and tobramycin (n = 2; 11.0%).

Therefore, susceptibility rates for amikacin changed from 84.2% (n = 16) to 100% (n = 19); for tobramycin changed from 68.4% (n = 13) to 79.0% (n = 15) and for nalidixic acid from 73.7% (n = 14) to 79.0% (n = 15).

Patients characteristics, antimicrobial therapy, underlying disease and outcome are described in Table 2. Patients were hospitalized either in medical, surgical wards or in the emergency department. Most of them were female (52.6%), had oncological diagnosis and almost all (except for one) received at least one carbapenem as treatment, however, the outcomes were poor and 68,6% resulted in death (n = 13, 68,6%). The mean of days at ICU were 8.7 days, with a minimum of zero days and maximum of 31 days.

Table 2
Clinical characteristics of patients with positive *Acinetobacter baumannii* isolates

Isolate	Date of isolation	Sex	Ward	Underlying disease	Site of Isolation	Treatment	Outcome	Hospitalization date	Antibiotic Therapy Date	Days at ICU	Proced
2	10.02.2015	Male	ED	Nasal Lymphoma	Blood Culture	AMC, MEM, TZP, VAN, COL	Dead	25/01/2016	26/01/2016	14	Lima
10	09.12.2015	Male	MOD	Acute Lymphoblastic Leukemia	Blood Culture	MEM, TZP, VAN, MTZ, LZ, COL	Cure	20/08/2015	25/09/2015	0	Lima
20	15.12.16	Female	MOD	Gastric Non-Hodgkin T Lymphoma	Blood Culture	MEM, SXT, VAN	Dead	19/04/2015	21/04/2015	30	Ancash
21	15.12.2016	Female	MOD	Acute Lymphoblastic Leukemia	Blood Culture	MEM, SXT, VAN	Dead	8/02/2016	9/12/2016	0	Ica
22	27.09.2015	Male	PD	T Cell Acute Lymphoblastic Leukemia	Blood Culture	MEM, SXT, VAN, COL	Dead	24/09/2015	27/09/2015	14	San Ma
23	15.07.2015	Male	MOD	Acute Myeloid Leukemia (M1)	Blood Culture	MEM, MTZ, VAN	Dead	2/07/2015	3/07/2015	0	Lima
24	09.03.2015	Male	TS	Diffuse Large B-Cell Non-Hodgkin Lymphoma	Blood Culture	MEM, MTZ, LZ, VAN	Dead	9/02/2015	1/03/2015	12	Lima
25	30.03.2015	Female	MOD	Acute Myeloid Leukemia	Blood Culture	MEM, TZP, VAN	Dead	24/03/2015	24/03/2015	2	Cajama
26	05.08.2015	Male	ED	Scrotal Cancer	Soft Tissue	MTZ, CIP	Cure	15/07/2015	15/07/2015	0	Cuzco
27	01.03.2015	Male	MOD	Xanthoastrocytoma	Blood Culture	MEM, VAN	Cure	31/12/2014	14/01/2015	2	Amazo
28	23.11.2015	Male	HN	Diffuse Large B-Cell Non-Hodgkin Lymphoma	Blood Culture	FEP	Cure	25/10/2015	25/10/2015	0	Lima
29	17.03.2015	Female	PD	Acute Lymphoblastic Leukemia	Blood Culture	MEM, TZP	Cure	9/03/2016	14/03/2016	0	La Libe
30	04.04.2015	Female	MOD	Diffuse Large B-Cell Non-Hodgkin Lymphoma	Bronchial Secretion	MEM, TZP, SXT, LZ	Dead	25/03/2015	27/03/2014	12	Lima
39	05.07.2015	Female	PD	Acute Lymphoblastic Leukemia	Blood Culture	MEM, STX, VAN	Dead	30/05/2014	30/05/2014	20	Tacna
41	27.06.2014	Female	MOD	Mixed Phenotype Acute Leukemia	Blood Culture	MEM, TZP, VAN	Dead	7/06/2014	7/06/2014	5	Apurim
46	19.03.2015	Female	PD	B Cell Acute Lymphoblastic Leukemia	Blood Culture	MEM, MTZ, VAN	Dead	6/02/2015	15/02/2015	0	Puno
50	23.03.2015	Female	ED	Cavernous sinus tumor	Blood Culture	MEM, VAN	Dead	20/02/2015	20/02/2015	21	Lima
54	01.06.2015	Male	ED	Prostate Cancer	Bronchial Secretion	MEM, COL	Cure	18/05/2015	7/06/2015	31	Junin
55	02.09.2015	Female	ED	Colorrectal Cancer and Endometrial Adenocarcinoma	Blood Culture	MEM, VAN, LZ	Dead	14/07/2015	14/07/2015	3	Junin

ED, Emergency Department; MOD, Medical Oncology Department; PD, Pediatrics Department; HN, Head and Neck Surgery Department; TS, Thoracic Surgery Department. AMC, amoxicillin-clavulanic acid; MEM, meropenem; TZP, piperacillin/tazobactam; VAN, vancomycin; MTZ, metronidazole; COL, colistin; CIP, ciprofloxacin, LZD, linezolide; F

Discussion

In this study, efflux pumps expression was detected in sixteen of the strains (84.2%) as the MIC decreased in at least one antimicrobial compound, after the addition of PAβN. Eight strains (42.1%) had MIC reduction for at least two antimicrobials and one isolate (5.3%) showed MIC decrease for three antibiotic

agents. Susceptibility profiles of the *Acinetobacter baumannii* strains included in this study are described in Table 1. Amikacin and nalidixic acid action were enhanced with PABN in seven strains a four-fold decrease of the MIC. Some strains also increased their susceptibility for levofloxacin (n = 5), ciprofloxacin (n = 4) and tobramycin (n = 2), reflecting the broad spectrum of PAβN. In addition, eight strains changed their susceptibility profile by MIC according to CLSI reference values (28).

This is the first assessment of efflux pumps inhibitors in *Acinetobacter baumannii* strains in Peru, besides worldwide literature of PAβN in *Acinetobacter* spp. strains is still limited.

Consistent with the findings of Peleg et al(31) and Valentine et al (30) the ciprofloxacin MICs for most of the *A. baumannii* isolates (7/19) did not change more than fourfold in the presence of PAβN. This was also the case for the other antimicrobial agents in the present study. Results from a study of 103 Acinetobacter isolates in Tehran showed a 40% lower ciprofloxacin MIC with the addition of PAβN, but only 6,10% changed to susceptible MIC values (32) In addition, Golanbar et al. also demonstrated a reduction on MIC while using PAβN (33), while Ribera et al. did not observed that an effect on MIC of ciprofloxacin in addition to PAβN (34). Overall, in many studies, including the present one, the inhibitory effect of PAβN has been demonstrated (32, 35).

According to Coyne et al, comparison of the resistance levels of a clinical *A. baumannii* MDR strains confirms that efflux is a major factor for resistance to various drug classes, including β-lactams, chloramphenicol, macrolides, tetracyclines, and aminoglycosides, with high-level resistance to fluoroquinolones requiring additional mechanisms, such as alteration of DNA type II isomerases (17). Cheng et al found that fluoroquinolones use predispose to a high colonization density of MDR in nasal and fecal specimens(36). Efflux pumps have an important role on developing *Acinetobacter* spp antimicrobial resistance, along with overexpression of AmpC, β-lactamases or carbapenemases (11). This is also the case of the analyzed strains, as they expressed oxacillinases (OXA-23, OXA-24 and OXA-143) in a previous study (25) along with efflux pumps.

Patients characteristics were assessed in Table 2. All had oncologic diagnosis, mainly leukemia and other hematological malignancies, with most having an ICU staying, broad spectrum antibiotic (mainly imipenem) and over 50 years old. They preceded from different country regions and could spread dangerous clones across the country(25). Previously described features have already been identified as MDR *Acinetobacter baumannii* risk factors in other studies (7, 8) and their identification warns about the requirement for appropriate antimicrobial surveillance programs and infection control standards (1, 9).

The results of the present study indicate that efflux pumps have a role in conferring MDR resistance in *A. baumannii* clinical isolates. Thus, efforts should be aimed at effectively detecting this pathogen and its resistance mechanisms in order to improve healthcare standards. Moreover, further research is needed to find more suitable, new compounds and effective therapies (13) against *A. baumannii*. Updated available data particularly support the development of efflux pump inhibitors for use in combination with antibiotics (23). Further studies are required to assess the efficacy and safety of such compounds alongside current therapy in order to decrease the burden of disease attributable to this successful pathogen.

In conclusion, High resistance rates were detected, decreasing with PAβN as its effect of EPI. Efflux pumps still represent an important mechanism of antimicrobial resistance in *Acinetobacter bumannii* strains and focusing at inhibitory effects of some substances, including PAβN, could provide insight as complementary therapy or new antimicrobials in future research.

Limitations

The study had the limitation that only some strains were recovered from the collection of the 2014 to 2016 outbreak. The activity of efflux pumps inhibitor was only evaluated for PAβN, it could be evaluated for other EPI in future studies.

Abbreviations

A. bumannii: Acinetobacter bumannii; PACE: proteobacterial antimicrobial compound efflux; MIC: Minimun inhibitory concentration; PAβN: arginine-β-naphthylamide; PCR: polymerase chain reaction.

Declarations

Ethics approval and consent to participate

The study protocol was approved by the Research Ethics Board of the *Instituto de Investigación Nutricional* (IIN). The samples of clinical laboratory isolates were obtained in the context of infection surveillance regulated by the Committee for the Control and Prevention of Intrahospital Infections of the *Instituto Nacional de Enfermedades Neoplásicas* in accordance with Technical Standard No. 753-2004 / Ministry of Health of Peru. and with the International Ethical Guidelines for Health-related Research Involving Humans. In-hospital infections (IIH) also called - Health care-associated infections (HCAIs) are a Public Health problem and under these provisions, the collection of samples was exempt from informed consent. Patient information was coded when it was collected to ensure anonymity and confidentiality and its characteristics were evaluated from their clinical records.

Consent to publish

Not Applicable

Availability of data and materials

Abstraction format used in the study and dataset are available and accessible from the corresponding author upon request in the link: https://figshare.com/s/d1cddba64d9769ffe92b

Conflicts of interest

On behalf of all authors, the corresponding author states that there are no conflicts of interest or funding related to this study

Funding

This study was supported by CONCYTEC Peru, under the contract N° 164-2016-FONDECYT, Lima, Peru. Incentive for Research of the Universidad Peruana de Ciencias Aplicadas (Nº UPC-C05-2016), Lima, Peru. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Authors' contributions

SPR, SLB and JdVM designed the study protocol. SPR, SLB, IPT and MAAL performed the microbiological analysis. JdVM, WSC and MAAL: responsible for obtaining funding and laboratory work supervision. SPR, WVT and FBB was responsible for the clinical assessment, samples collection and database completion. SPR, SLB, WSC and JdVM drafted the manuscript. All authors critically revised the manuscript for intellectual content. All authors read and approved the final manuscript.

Acknowledgments

We thank the staff of the health network from *Instituto Nacional de Enfermedades Neoplásicas*, Lima- Peru.

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